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Skeletal Development in Embryonic Quail. *

* The original title: "Skeletal Development in Embryonic Quail on the International Space Station" has been changed to indicate that the actual flight occurred on STS-108 in a mid-deck locker, not on the ISS as originally intended.

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I.Introduction

A.Abstract: The purpose of this study is to define the effects of spaceflight on embryonic skeletal development. The justification for such a study is two fold: (1) One major goal of NASA research is eventually to study the egg-to-egg cycle of development and determine the effect of microgravity on the many stages of development included in such a cycle. (2) The embryogenesis of skeletal development also occurs in the adult skeleton during fracture repair. The information learned from the study of embryogenesis of bone and cartilage will be the basis for future studies of fracture repair in microgravity environments.

Previous study of skeletal development occurred in the MIR21/NASA2 flight, where embryonic quail were analyzed at 7,10 and 14 days of development. In these studies we found by electron microscopy and also x-ray microanalysis, that there was a delay in mineralization at the forming bony collar in the 7 and 10 day embryos. By the time the embryos approached hatching, the bone structure, the mineral content, and bone length were all normal compared to ground controls. The present study is designed to investigate these earlier findings in more detail, to study the tissues in more detail at the cellular level, and to impose a 1-G centrifuge force during spaceflight to determine if a gravitational force influences embryogenesis during spaceflight.

B.Hypothesis: Previous studies of spaceflight and microgravity on adult skeletal tissues indicate that there is muscle and bone loss due to microgravity. These studies were carried out on subjects who went into space with formed skeletal tissues which made it difficult to determine any change which was imposed by microgravity. Our hypothesis was : (1) Spaceflight and microgravity would not affect the initial cartilage anlage formation of the skeletal elements. We had no reason to expect that initial embryogenesis of the skeleton, which was a cartilage scaffold of the developing limbs, would be affected by spaceflight. (2) Based on previous studies, we could expect microgravity to inhibit either (a) the conversion of cartilage to bone or (b) the mineralization of the early bone matrix .

C. Objectives of Investigation: This investigation was unique in that we had access to the newly developed Animal Development Facility (ADF) developed by Ames Research Center and SHOT (Space Hardware Optimization Technology, Inc; Greenville, Indiana). This hardware provided us with egg incubator conditions utilizing two separate carousels, one of which was rotated during the spaceflight to provide a 1-G environment for a portion of the egg population. Using this particular hardware, our objectives were:

1. Compare development of embryos in presence or absence of 1-G during spaceflight.
2. Compare development of different bones (eg. calvaria vs tibia) which form by different methods of embryogenesis; ie, endochondral vs intramembranous formation.

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3. Evaluate cartilage growth and initial bone matrix deposition in flight and ground control embryos (eg. proteoglycan stains, fibronectin, decorin, and type X collagen).
4. Evaluate bone matrix formation and matrix content (eg. Type I collagen, osteocalcin, decorin, polarized light for lamellar formation).
5. Compare bone cell activity during bone formation and relate to spaceflight conditions. This involves electron microscopy, light microscopic immunocytochemistry, and histo-morphometry. The immunostaining will utilize antibodies to cellular procollagen (type-I), BMPs, CBFa, and cyclin D.
6. Compare mineralization of developing bone matrix by FTIR and EDAX x-ray analysis..

D.Background/History of Project: In a previous study of quail development onboard the MIR space station (MIR 21/ NASA 2) we noted that there was a delay in the conversion of cartilage to bone and in the mineralization of newly forming bone matrix. This flight experiment consisted of quail embryos, preserved by alcohol fixation during flight, at developmental days 7, 10,14 and 16. At dissection, the limbs showed no difference in limb length when ground controls and flight animals were compared. However, by electron microscopy we showed a small delay in mineralization at the bony collar in flight animals. Using EDAX (energy dispersive analysis by x-ray) analysis we could find a reduced Ca/P ratio at embryonic day 10 however there were no differences at day 16. Fourier transform infrared analysis (FTIR) showed some differences in the bone crystal due to spaceflight, however most differences seen at early development were not present at the time of hatch (approx day 16 of incubation).

Previous studies of rodent bone indicates that bone loss occurs during spaceflight (Wronski, et al, 1987; Doty et al, 1992). A study by Turner et al (1995) suggested that reduced bone formation in rats can occur as early as 4-10 days of spaceflight. Vico et al (1991) in a 7 day flight (Biocosmos 1667), Evans et al (1998) on a 14 day flight (SLS-2) , and Zerath et al (2002) on a 14 day flight (Bion 11) showed that reduced bone formation was the most apparent response to spaceflight in bone. Bone resorption did not seem to be the major factor in young growing bone however it may play a role in bone loss in adults (Parfitt, 1981)

Studies of spaceflight effects on embryos seems to be very limited. Suda (1998) did not demonstrate any effect on chick embryogenesis or bone formation after a 7 day flight. And Kawashima et al (1995) indicated that 7 days of spaceflight followed by post-incubation on earth resulted in no effect on chick bone development. It should be pointed out here that these were studies measuring fairly gross responses and no studies were carried out at the cellular level looking for change in cell or metabolic activity.

II. Methods/Research Operations:

General methods used for this study:

Electron Microscopy
Light Microscopy
Immunocytochemistry
Fourier Transform InfraRed Microscopy (FTIR)
Energy Dispersive X-ray Microanalysis (EDAX)
Histomorphometry

A. Discussion of Methods:

General tissue collection. Eggs at development days 4 and 7 were fixed during flight by an automatic injection of fixative using the ADF hardware. A group of 3 eggs at day 4 and 6 eggs at day 7 were injected from each of 4 groups (ADF flight, 1-G; ADF flight, 0-G; ADF

ground control, centrifuge; ADF ground control, no centrifuge). The day 4 eggs contained no useful skeletal elements and were not used for this study. The day 12 eggs were dissected into various tissues and preserved for later study. The legs were cleaned of most muscle and skin and preserved in 4% paraformaldehyde fixative in 0.05M cacodylate buffer, pH 7.4 for 24 hours. The wings were preserved in 90% alcohol and maintained in alcohol for shipment and storage. Calvaria and mandible were collected as samples of flat bone and fixed in aldehyde fixative similar to the long bones.

Electron microscopy: Tissue samples fixed in aldehyde were dissected into small pieces of bone from the distal tibia and the midshaft. These samples were embedded in Epon or Spurrs resin for thin sectioning for transmission electron microscopy. These same samples are to be used for EDAX analysis of Ca/P values and related to the fine structure morphology. Some samples will be studied by scanning electron microscopy coupled with EDAX analysis for Ca/P values and comparisons between samples.

Light Microscopy: Fixed samples of long bones and calvaria/mandibles from each of the four major groups were decalcified in 10% EDTA in 0.05M Tris buffer, ph 7.4 for 1 week. These tissues were processed for paraffin embedding, sectioned and stained. All slides were stained for H&E and Trichrome for morphology. Alcian blue staining and Saffranin O were used to differentiate cartilage from bone and surrounding tissues.

Immunocytochemistry: Antibodies and their suppliers were: Type I and Type II collagen: Calbiochem. Collagen Type X and Procollagen, type I: Developmental Studies Hybridoma Bank. Cathepsin K: Santa Cruz. Osteocalcin: Zymed. The various BMP antibodies from Santa Cruz (BMP 2,4,&7) and Indian Hedgehog antibody did not work on this quail tissue. The primary antibody was used at dilution of 1:100 or 1:200 with overnight incubation at 4C. Secondary antibodies conjugated to peroxidase were applied for 1 hour at room temperature, followed by diaminobenzidine staining for 10-20 minutes. Sections were counterstained with hematoxylin.

Fourier Transform Infrared Microspectroscopy: Calcified tissues either embedded in polyethyl-methyl methacrylate or Spurs resin were sectioned at 1- 3 microns thickness and placed onto barium floride infrared windows. These sections will be analyzed using a Bio-Rad FTS infrared microscope with a detector undergoing nitrogen purge during analysis. Infrared spectra are collected from 20X20 micron spot on the tissue. From the collected spectra the mineral content, crystallinity of the bone mineral, and the mineral/matrix ratio will be determined. Spectra are collected from different areas of a single bone sample to compare between the major groups of quail embryos, and different bones will be analyzed from the same embryo.

Energy Dispersive X-ray Microanalysis (EDAX): (a) Samples for transmission electron microscopy (TEM) were sectioned at 0.1microns thick from calcified tissues embedded in Spurrs or Epon resin. Calcific deposits may be visualized without staining in the TEM because of the inherent density of the deposits. The 80Kv accelerating voltage of the electron beam generated x-rays from the sample which are collected through a thin window detector and analyzed by their specific energies. This permits a specific analysis for the presence or absence of calcium and phosphorus and their relative abundance in a volume of tissue approximated by the size of the beam diameter (10-100 angstroms) and the tissue thickness. (b) Samples for scanning electron microscopy (SEM) are air dried, coated with a thin layer of carbon, and analyzed similarly as the method in (a). However, these are intact whole limbs and not sections, so the analyzed volume is larger and the electron beam voltages are lower (15-20 Kv). These values represented the entire bony collar rather than a single locus on the tissue as seen by TEM. These Ca/P ratios for the entire limb are compared to the values from single locations. Also different limbs will be compared to each other among the four groups and long bones are compared to flat

bones to determine whether the method of tissue development resulted in any change in calcification.

B) List and Description of all Functional Objectives

ADF Functional Objectives:

FO1: Activate the ADF Incubation Mode (transition temperature from 13°C to 38.5°C and initiate centrifuge) of the ADF as soon after launch as possible.

FO2: Check ADF air inlet for obstruction, record environment data from ADF display and call down data.

FO3: ADF performed automated fixations at Incubation Days (ID) 4 and 7.

FO4: Activate ADF Idle Mode which maintains the environmental parameters for incubation, but stops the centrifuge from spinning for orbiter re-entry.

Session Chart:

Session Name	FO#	Hardware	Scheduled Day	Actual Day	Activity/Samples collected
Increment 4/UF-1					
ADF Incubate Mode	1	ADF	Day 1 12/5/01	Day 1 Dec. 5	ADF transitioned from 13°C to 38.5 °C
ADF Status Check	2	ADF	Twice Daily	Nearly twice a day	ADF environ. data called down
ID4[1] Fixation	3	ADF	N/A	Automated 96 hrs. post-inc. mode	3 eggs/ carousel were fixed with automated system
ID7 Fixation	3	ADF	N/A	Automated 168 hrs. post-inc. mode	6 eggs/ carousel were fixed with automated system
ADF Idle Mode	4	ADF	Day 11 12/15/01	Day 13 12/17/01	Centrifuge rotation stopped

1 – Incubation Day (ID) 4

C.) List of Hardware Items:

Item	Preflight (Pr) Inflight (I) Postflight (Po)	Owner	Description
1) Avian Development Facility	Pr, I, Po	SHOT/Ames	Flight hardware; egg incubator with contained centrifuge.
2) Laboratory facility	Pr, I, Po	KSC/Ames	Complete lab for egg incubation and embryo dissections.
3) Transmission electron microscope	Po	PI	Philips CM-12, FEI, Inc.
4) Scanning electron microscope	Po	PI	Quanta , FEI, Inc.
5) Nikon microscope with morphometry software,computers and video cameras	Po	PI	MicrophotFXA, Nikon
Software morphometry packages	Po	PI	Bioquant and Universal Imaging, Downingtown,PA.
6) Tissue processor and embedding	Po	PI	VIP processor, Belair Ins. Reichart Jung, Germany.
7) Microtome for sectioning calcified tissues for light microscopy and morphometry.	Po	PI	Leica, SM2500, MOC,Inc.

III. Results.

A) Anomalies: None noted.

B) Completeness/Quality of Data: The measured data eventually is evaluated on a statistical basis for one-tailed or two-tailed analysis of significance. Therefore the "n" or number of samples is critical. Because the overall yield of developed embryos was low (*Day 12:*Flight, 0xG, 4 of 9 embryos; Flight, 1XG, 6 of 9 embryos; GC, no spin, 6 of 9 embryos; GC, spin, 3 of 9 embryos. *Day 7:* 4 of 6; 3 of 6; 5 of 6; and 2 of 6 --in same grouping as day 12) the significance of any suggested changes were difficult to demonstrate.

C) Graphs of Results:

1) Embryo whole body weight. Chart gives data for only 12 day embryos.

TS-108 Embryo Body Weights (12 Day embryos)

			Grams:
ADF Flight:	**1XG**		3.42
			3.2
			3.75
			3.13
			3.3
			3.4
		Mean/SD	3.37+/- 0.22
	0XG		2.17
			3.61
			1.8
			2
		Mean/SD	2.40+/- 0.82
ADF Ground Control			
	No spin		3.15
			2.82
			3.3
			3.92
			3.47
			3.55
		Mean/SD	3.37+/- 0.37
	Spin		3.35
			3.11
			3.34
		Mean/SD	3.27+/- 0.14
Incubator Ground Control			2.3
	No spin		2.3
			2.48
			2.13
			2.49
			2.19
			2.39
			2.11
			2.32
		Mean/SD	2.31+/- 0.14

Stastical Comparisons:

ADF, Flight,1XG >>
ADF,Flight, 0XG
P=0.01

ADF, Flight, 1XG >>
Incubator GC
P<0.001

ADF Ground Control, no- spin>>
Incubator, GC
P<0.0001

Other groups not significantly different..

2) Type X collagen staining of cartilage anlage, corrected by differences in leg lengths among embryos. Staining intensity=optical density (OD) of stain. Length shown in mm.

EGG NUMBER	Leg Length (mm)	Collagen X	Collagen X / lenght	Group Average
		OD (-bkg)	OD/mm	
Faxitron # 601, flt 1G, 01-29-02, S02-59	8.40	58.75	7.00	6.73
Faxitron # 629, flt 1G, 01-29-02, S02-60	7.30	49.01	6.71	
Faxitron #663, flt 1G, 01-29-02, S02-61	7.06	38.89	5.51	
Faxitron # 664, flt 1G, 01-29-02, S02-62	7.38	55.94	7.58	
Faxitron # 665, flt 1G, 01-29-02, S02-63	7.75	60.52	7.81	
Faxitron # 673, flt 1G, 01-29-02, S02-64	7.59	43.91	5.79	
Faxitron # 642,flt 0G , 01-29-02, S02-65	5.30	38.86	7.34	7.10
Faxitron #659, flt 0G , 01-29-02, S02-66	6.58	42.76	6.49	
Faxitron # 729,flt 0G , 01-29-02, S02-67	5.99	46.57	7.78	
Faxitron # 758,flt 0G , 01-29-02, S02-68	7.37	50.12	6.80	
Faxitron #839, GC no spin, 01-29-02, S02-72	7.99	60.29	7.55	5.71
Faxitron #911, GC no spin, 01-29-02, S02-73	7.47	30.31	4.06	
Faxitron # 917, GC no spin, 01-29-02, S02-74	5.95	38.36	6.45	
Faxitron # 922, GC no spin, 01-29-02, S02-75	8.05	35.40	4.40	
Faxitron # 930, GC no spin, 01-29-02, S02-76	8.00	40.04	5.00	
Faxitron # 945, GC no spin, 01-29-02, S02-77	6.08	41.31	6.79	
S02-495B, Incubator control	5.60	47.71	8.52	7.63
S02-496B, Incubator Control	6.00	47.48	7.91	
S02-497B, Incubator Control	7.60	50.02	6.58	
S02-498B, Incubator Control	6.80	45.32	6.66	
S02-499B, Incubator Control	6.30	52.36	8.31	
S02-500B, Incubator Control	6.50	53.66	8.26	
S02-501B, Incubator Control	7.40	53.02	7.16	

Faxitron # 829, GC 1G, 01-29-02, S02-69	7.18
Faxitron # 841, GC 1G, 01-29-02, S02-70	7.38
Faxitron # 888, GC 1G, 01-29-02, S02-71	6.11

3) Osteocalcin staining of bone matrix, corrected by adjusting for variation in leg length among 12 day embryos. Stain intensity measured as optical density. Leg length measured in mm.

EGG NUMBER	Leg Length (mm)	Osteocalcin	Osteocalcin / lenght	Group Average	STD
		(OD) - bkg	OD/mm		
Faxitron # 601, flt 1G, 01-29-02, S02-59	8.40	84.74	10.09	8.90	1.39
Faxitron # 629, flt 1G, 01-29-02, S02-60	7.30	59.49	8.14		
Faxitron #663, flt 1G, 01-29-02, S02-61	7.06	77.95	11.04		
Faxitron # 664, flt 1G, 01-29-02, S02-62	7.38	54.52	7.39		
Faxitron # 665, flt 1G, 01-29-02, S02-63	7.75	62.67	8.09		
Faxitron # 673, flt 1G, 01-29-02, S02-64	7.59	65.61	8.65		
Faxitron # 642,flt 0G , 01-29-02, S02-65	5.30	56.10	10.59	9.31	1.92
Faxitron #659, flt 0G , 01-29-02, S02-66	6.58	44.10	6.70		
Faxitron # 729,flt 0G , 01-29-02, S02-67	5.99	65.27	10.90		
Faxitron # 758,flt 0G , 01-29-02, S02-68	7.37	66.82	9.07		
Faxitron #839,GC no spin, 01-29-02, S02-72	7.99	61.01	7.64	8.55	1.49
Faxitron #911, GC no spin, 01-29-02, S02-73	7.47	48.50	6.50		
Faxitron # 917, GC no spin, 01-29-02, S02-74	5.95	53.69	9.02		
Faxitron # 922, GC no spin, 01-29-02, S02-75	8.05	66.59	8.27		

Faxitron # 930, GC no spin, 01-29-02, S02-76	8.00	71.84	8.98		
S02-497B, Incubator Control	7.60	60.12	7.91		
S02-495B, Incubator control	5.60	46.70	8.34	9.22	1.15
S02-498B, Incubator Control	6.80	61.07	8.98		
S02-499B, Incubator Control	6.30	61.93	9.83		
S02-500B, Incubator Control	6.50	74.40	11.45		
S02-501B, Incubator Control	7.40	67.53	9.13		

4) Procollagen Type I staining of osteoblasts forming bony collar; corrected for variation in leg length. Stain intensity measured as optical density. Leg length measured in mm.

EGG NUMBER	Leg Length (mm)	Procollagen I	Procollagen I / lenght	Group Average
		(OD) - bkg	OD/mm	
Faxitron # 601, flt 1G, 01-29-02, S02-59	8.40	155.95	18.58	18.93
Faxitron # 629, flt 1G, 01-29-02, S02-60	7.30	126.40	17.30	
Faxitron #663, flt 1G, 01-29-02, S02-61	7.06	114.50	16.22	
Faxitron # 664, flt 1G, 01-29-02, S02-62	7.38	173.64	23.52	
Faxitron # 665, flt 1G, 01-29-02, S02-63	7.75	140.01	18.07	
Faxitron # 673, flt 1G, 01-29-02, S02-64	7.59	150.69	19.86	
Faxitron # 642,flt 0G , 01-29-02, S02-65	5.30	154.47	29.17	21.14
Faxitron #659, flt 0G , 01-29-02, S02-66	6.58	124.36	18.89	
Faxitron # 729,flt 0G , 01-29-02, S02-67	5.99	125.71	20.99	
Faxitron # 758,flt 0G , 01-29-02, S02-68	7.37	114.16	15.49	
Faxitron #839, Gc no spin, 01-29-02, S02-72	7.99	135.67	16.98	27.48
Faxitron #911, GC no spin, 01-29-02, S02-73	7.47	209.04	28.00	
Faxitron # 917, GC no spin , 01-29-02, S02-74	5.95	203.83	34.26	
Faxitron # 922, GC no spin, 01-29-02, S02-75	8.05	206.15	25.60	
Faxitron # 930, GC no spin, 01-29-02, S02-76	8.00	155.52	19.43	
Faxitron # 945, GC no spin, 01-29-02, S02-77	6.08	246.98	40.62	
S02-495B, Incubator control	5.60	200.93	35.88	30.18
S02-496B, Incubator Control	6.00	213.46	35.58	
S02-497B, Incubator Control	7.60	189.14	24.89	
S02-498B, Incubator Control	6.80	187.82	27.62	
S02-499B, Incubator Control	6.30	189.36	30.06	
S02-500B, Incubator Control	6.50	192.72	29.65	
S02-501B, Incubator Control	7.40	204.10	27.58	

5) Statistical comparisons between groups and between various immunostains:

(a) Leg length differences:

ADF, flight, 1XG = 7.58 mm , n=6, SD=0.47

ADF, flight, 0XG = 6.31 mm, n=4, SD=0.88

ADF,flight,1XG >> ADF, flight, 0XG : p=0.008

ADF, flight, 1XG = 7.58 mm, n=6, SD=0.47

Incubator ground control, no spin = 6.6 mm, n=7, SD=0.72

ADF,flight,1XG>> Incubator control,no spin.: p=0.008

ADF, flight, 0XG = 6.31 mm, n=4, SD=0.88

ADF, ground control, no spin = 7.26 , n=6, SD=0.99

ADF,ground control,no spin> ADF,flight, 0XG: p=0.08

(b) Type X collagen staining of hypertropic cartilage, corrected for leg length differences:

ADF, flight, 1xG = 6.73 OD/mm, n=6, SD=0.93

ADF, flight, 0XG = 7.10 OD/mm, n=4, SD=0.57

ADF, ground controls, no spin = 5.71 OD/mm, n=6, SD=1.42
Incubator ground controls, no spin = 7.63 OD/mm, n=7, SD=0.81
No significance in differences between groups.

© Osteocalcin immunostaining of bone matrix (does not include cellular staining):
ADF, flight, 1XG = 8.90 OD/mm, n=6, SD=1.39
ADF, flight, 0XG = 9.31 OD/mm, n=4, SD=1.92
ADF, ground control, no spin = 8.55 OD/mm, n=6, SD=1.49
ADF, incubator control, no spin = 9.22 OD/mm, n=7, SD1.15
No significance in differences between groups.

(d) Procollagen Type I immunostaining of osteoblasts adjacent to bone collar:
ADF, flight, 1XG = 18.93 :OD/mm, n=6, SD=2.56
ADF, flight, 0XG = 21.14 OD/mm, n=4, SD=5.82
ADF, ground control,no spin= 27.48 OD/mm, n=6, SD=8.91
Incubator control, no spin = 30.18 OD/mm, n=7, SD=4.15
ADF,ground control, no spin > ADF, flight, 1XG: p=0.02
Incubator control, no spin >> ADF, flight, 1XG: p<0.0001
ADF, ground control, no spin >Combined ADF flight, 1xG and 0XG: p=0.016

D) Photographs: Photos of immunostaining for the various antibodies are available in a Photo Appendix as separate attachments.

E) Status of Data Analysis: There are some aspects of this study which are incomplete for the following reasons:

a) There is no presentation of D7 embryo data because the number of samples collected from these embryos was too small to carry out any statistical analysis.
For example: The staining for procollagen type I in the D7 embryos gave the following results: ADF, flight, 1XG: n=4, OD=167.6
ADF, flight, 0XG: n=3, OD=102.2
Incubator, ground control, no spin: n=3, OD=197.3
These results suggest that collagen synthesis may have been reduced at Day 7 due to microgravity. This was not seen at Day 12.. However when we carry out the mineral analysis we need to carefully compare day 7 with day 12, especially among the flight groups..

b) We did not include the group: *ADF,Ground Control, with spin*, for comparative analysis because there were relatively few embryos from this group and statistical analysis would not be possible.

c) We have not begun the analysis of mineral content by FTIR or combined EM and EDAX analysis. We first wanted to find out whether there was any significant developmental differences between the groups which would help us focus on specific tissues for the mineral analyses. These mineral analyses will be completed as we narrow our search for the proper group or specific areas to study. The study of mineralization will be confined to small areas for analysis and therefore we need to have in-depth background information for making choices of areas to analyze and areas for making comparisons.

F) Concluding Investigative Results:

a) The variation in body weights of these embryos directly correlated with the leg length measurements. Therefore we could not place much importance on leg length as a response to spaceflight, since the real effect might have been on whole body size. The conclusions from body size and leg length indicate that the 1XG centrifuge in the ADF resulted in larger embryos and better development compared to the 0XG embryos in the same hardware. Fortunately, the Incubator Controls were the same body size as the ADF,0XG, flight embryos so we could compare flight embryos by size to one of the two ground control groups. In general, the ADF,flight, 1XG embryos were larger (and legs longer) than either the ADF,flight, 0XG or the Incubator controls. And the ADF,flight, 0XG and the Incubator Controls were statistically the same size.

b) The ADF, ground control, plus spin (whose purpose was to mimic the centrifuged group in space) were not used in these evaluations. The number of embryos from this group was very low and they were also exposed to hyper-g forces as they were centrifuged in the 1XG environment of earth. So these embryos were of no use in this study.

c) Adjusting for embryo size and differences in leg length, there was no difference in cartilage staining for the presence of Type X collagen in hypertropic chondrocytes. This indicated that cartilage differentiation was not affected by spaceflight, in the day 12 embryos. This also indicated that vascular invasion of cartilage and growth in length was probably not affected by spaceflight. If this latter had occurred, we might expect the hypertrophic cartilage cells to show some effect either by lack of differentiation, an enlarged zone of undifferentiated cartilage cells, or a lack of Type X collagen. None of these were seen in the day 12 embryos.

d) Adjusting for leg length differences, the staining of bone for presence of osteocalcin was not different between the groups. This suggests that mineralization of bone was not affected by spaceflight. Again, these are results of the day 12 embryos and we do not have data on the day 7 embryos because of lack of tissue. However, we should have enough tissue for the mineral analysis of both day 7 and day 12, to answer this question more directly.

e) Adjusting for differences in leg length (and embryo size) procollagen Type I showed a decrease in osteoblasts content (ie, optical density of staining) around the bony collar in the ADF,flight, 1XG animals vs the ADF ground control and vs the Incubator controls. If we combine data for the ADF flight samples (both 1XG and 0XG) to increase the value of "n", the ADF, ground control contained greater staining in their osteoblasts compared to all the flight animals ($p=0.016$).

G) Conclusions:

1) The ADF hardware coupled with its 1XG spinning carousel, improved embryo viability and resulted in increased body weight and larger limbs than the 0XG flight embryos. This is based only on the D12 embryos since there were so few D7 samples in this study.
2) Measurements of Type X collagen and proteoglycan staining of cartilage in the Day 12 embryos indicates that spaceflight has no effect on cartilage during the early development stages.

3) In this study of the D12 embryos there was no effect of spaceflight on osteocalcin staining of bone matrix. Since osteocalcin in the matrix reflects the degree of mineralization, this would suggest mineralization is not affected. However, in our earlier study of MIR/NASA-2 we found some evidence of altered mineralization in the day 7 and day 10 embryos. In the present study we could not conclude anything from the day 7 embryos because of their small numbers. This conclusion will not be finalized until we carry out the direct mineral measurements on both the day 7 and day 12 tissue samples.

4) The procollagen Type I results are the most interesting data from this study, especially if we keep in mind that reduced new bone formation has been found in many previous studies in which mature animals were flown.

In this study, immunostaining for procollagen Type I, was used as an indicator of the collagen synthesizing activity of those osteoblasts which were forming the bony collar during embryogenesis of the long bones. This data indicated that the ground controls were more active in synthesizing collagen than either one of the two spaceflight groups, with or without the applied G force. These results could be a statistical problem in that the number of samples are small and further studies could produce a different result. However, if these data are correct, then spaceflight has an environmental component which can influence collagen synthesis which is not correctable by an applied 1XG force. Such environmental factors are probably numerous, but two important factors would be (a) radiation and (b) vibration. It could also be argued that both these factors would be more critical to the younger embryo compared to the day 12-14 embryo ready to hatch. The presence of one of these factors could explain the differences we find at early embryonic age but might not have the same effect in the older embryo.

These results related to osteoblast function will still have to be tempered by the results of the mineralization studies which are not complete. If there is no difference in mineral content among these groups, then the procollagen data loses much of its practical significance.

5) Finally it should be emphasized that the ADF hardware functioned extremely well for this type of study. The presence of the 1XG spinning carousel was critical to this project. This type of project needs to be carried out on ISS as we originally had planned, for several reasons: On ISS more samples could be collected for each time point. On ISS an egg-to-egg "generation" study could be carried out. On ISS, if radiation is a factor in embryogenesis, the effects of longer exposures could be determined.

(H) Earth Benefits: There may be earth benefits to the general study of embryogenesis in space but since I am not an embryologist, I cannot evaluate such potential benefits. However as someone who has studied bone physiology for 30 years, I can say that there is much to be learned from the embryogenesis of bone development which can influence our understanding of osteoporosis and fracture healing. For example, fracture healing consists of cartilage formation (callus), conversion of cartilage to a temporary bony stucture (similar to bony collar in embryogenesis), and new bone formation involving osteoblasts, collagen synthesis, etc. In osteoporosis the formation of new bone generally cannot be stimulated and the only treatment is to try and prevent loss of bone which already exists in the skeleton. But embryogenesis is largely

a bone forming system and the more that we can understand what drives that system, the more likely we will find a way to stimulate bone formation in the adult skeleton. The study of skeletal embryogenesis has much to offer at the basic science level which can eventually contribute to clinical treatment of skeletal deficiencies. The use of spaceflight or microgravity to perturb embryogenesis is one of the few methods we have to change the embryonic environment without destroying the embryo or creating an environment in which development is greatly different from normal.